Distribution, dynamics and *in situ* ecophysiology of *Crenarchaeota* in anaerobic wastewater treatment granular biofilms

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Abstract The archaeal communities of six anaerobic granular biofilm samples were surveyed for the occurrence of *Crenarchaeota*, using clone library and terminal restriction fragment length polymorphism (TRFLP) analysis. One of the samples, containing a high abundance of *Crenarchaeota*-like organisms, was used to inoculate anaerobic reactors for the treatment of a whey-based wastewater. TRFLP was used to monitor the *Crenarchaeota*-like species during the trial in response to various perturbations. FISH was employed to establish the spatial arrangement of *Archaea*, *Crenarchaeota* and *Bacteria* within biofilm architecture. Radioactive tracer experiments and micro-beta-imaging were used to describe substrate uptake patterns by members of the biofilm. The work represents a groundbreaking effort to investigate the role and *in situ* functionalism of *Crenarchaeota* and other groups within biotechnologically important granular biofilm-based ecosystems.

Keywords Anaerobic granular biofilm; beta-imaging; *Crenarchaeota*; ecophysiology; FISH; terminal restriction fragment length polymorphism

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Introduction

Crenarchaeotal phylogeny has fundamentally changed by the isolation of novel SSU rRNA gene clones from mesophilic and psychrophilic habitats. Quantification of rRNA abundance in oceanic waters indicates that these novel species constitute a significant proportion of the marine bacterioplankton. Indeed, rRNA gene sequences representing non-thermophilic clades of *Crenarchaeota* have been reported from mesophilic soils in disparate locations in the United States (Bintrim *et al.*, 1997), Finland (Jurgens and Saano, 1999) and Japan (Kudo *et al.*, 1997), and in granular biofilm from psychrophilic anaerobic bioreactors treating various wastewaters (Collins *et al.*, 2003, 2005; McHugh *et al.*, 2004). Notwithstanding the many reports documenting their presence in ambient environmental samples, the absence of non-thermophilic isolates has hindered a biochemical and physiological characterisation of these organisms. Thus, the actual ecological roles and specific physiological functions of these abundant and cosmopolitan *Crenarchaeota* remain a mystery. While the unusual properties of thermophilic *Crenarchaeota* have attracted the attention of both exobiologists wishing to study microbial evolution and biotechnology companies wishing to exploit the hyperthermotolerance of crenarchaeotal cellular enzymes, the widespread occurrence of non-thermophilic *Crenarchaeota*, particularly in engineered environments such as anaerobic digesters, means that the biotechnological significance and potential of these organisms should now be explored. This paper describes an investigation into the prevalence, population dynamics and *in situ* functionalism of *Crenarchaeota* in biofilm samples from low-temperature anaerobic wastewater treatment reactors. 16S rRNA clone library analysis, terminal restriction fragment length

polymorphism (TRFLP) and fluorescent *in situ* hybridisation (FISH) were applied in conjunction with radioactive tracer techniques and micro-beta-imaging to investigate substrate uptake patterns. This is the first application of this technology to anaerobic granular biofilm and represents an important advancement, both in terms of microbial ecology and efforts to describe the role and mechanisms of microorganisms involved in wastewater treatment.

Materials and methods

Biomass

Six anaerobic granular biofilm sludges, S1–S6, were obtained from various full- and pilot-scale biological wastewater treatment plants. S1 was collected from a full-scale UASB treatment plant at Carbery Milk Products, Ballineen, Co. Cork, Ireland; S2 was from a pilot-scale anaerobic filter-expanded granular sludge bed (AF-EGSB) reactor treating medium-strength (5 g COD l^{-1}) VFA-based wastewater; S3 and S4 were from laboratory-scale upflow anaerobic sludge bed (UASB) reactors used to treat acetate-based and propionate/butyrate/ethanol-based wastewaters, respectively; S5 and S6 were from full-scale internal circulation (IC) reactors at Archer Daniels Midland (ADM), Ringaskiddy, Co. Cork, Ireland and Carbery Milk Products, Co. Cork, respectively.

Reactor design and operation

Two pilot-scale EGSB reactors (R1 and R2) were each inoculated with 70 g VSS of the S6 particulate biofilm. The reactors were used for the stabilisation of a whey-based wastewater. The whey was obtained in dried form from a full-scale milk-processing plant and was made up to a final concentration of 10 g COD l^{-1} and 1 g COD l^{-1} for R1 and R2, respectively. The synthetic influent was buffered with $NaHCO_3$. R1 and R2 were initiated at an organic loading rate (OLR) of 5 and 0.5 kg COD m^{-3} d^{-1}, respectively and a hydraulic retention time (HRT) of 48 h. The OLR applied to R1 was increased to 10 kg COD m^{-3} d^{-1} (day 83) and 15 kg COD m^{-3} d^{-1} (day 167) by a stepwise reduction of the HRT to 24 h and 18 h, respectively. On day 470, the OLR applied to R1 was reduced to 7.5 kg COD m^{-3} d^{-1} in response to a decline in COD removal efficiency by lowering the COD concentration of the influent from 10 to 5 g l^{-1}, while maintaining the HRT at 18 h. The OLR applied to R2 was increased to 1 kg COD m^{-3} d^{-1} (day 83) and to 1.5 kg COD m^{-3} d^{-1} (day 167), by a stepwise reduction of the HRT to 24 h and 18 h, respectively. The initial operational temperature of both reactors was maintained at 20 °C and this was decreased during the trial to 18 °C, 16 °C, 14 °C and 12 °C on days 257, 340, 354 and 410, respectively. Effluent was recirculated through the systems to give a liquid upflow velocity of 5 m h^{-1}, which was increased in a stepwise manner for R1 and R2 to 7.5 m h^{-1}, 10 m h^{-1} and 12.5 m h^{-1} on days 4, 134 and 448 of the study, respectively.

DNA extraction, PCR-amplification and clone library analyses

Total genomic DNA was extracted from all six samples (S1–S6); archaeal 16S rRNA genes were amplified with forward primer 21F (5′-TTCCGGTTGATCCYGCCGGA-3′; Stackebrandt and Goodfellow, 1991) and reverse primer 958R (5′-YCCGGCGTTGAMTCCAATT-3′; DeLong, 1992); sequences were obtained from 16S rRNA gene clone libraries and phylogenetic reconstruction was carried out as described in detail by Collins *et al.* (2003).

TRFLP analysis

Biomass samples were collected from the granular sludge beds of the R1 and R2 reactors on days 21, 71, 218, 309, 354, 409 and 500, via a sampling port located near the base of

the reactor, from which DNA was extracted as before. Archaeal PCR products were attained for TRFLP analysis as described previously (Collins *et al.*, 2003).

FISH and confocal laser scanning microscopy

Briefly, samples for analysis were washed gently in phosphate buffered saline (PBS) three times and were fixed in 4% paraformaldehyde (Sigma) in PBS, at 4 °C for 6 h before exposure to 50% ethanol in PBS for 12 h at 4 °C. Granules were embedded in OCT freezing medium overnight at 4 °C. Serial sections (8 μm thick) were cut using a cryomicrotome and mounted on gelatine-coated slides. FISH was performed as described by Schramm *et al.* (1998). A hierarchical set of fluorescently labelled 16S rRNA-targeted oligonucleotide probes (Biomers.net, Germany) was used: (i) Arch915 (Stahl and Amann, 1991), specific for *Archaea*; (ii) Eub338 (Amann *et al.*, 1990), specific for *Bacteria*; (iii) Cren499 (Burggraf *et al.*, 1994), specific for most of the *Crenarchaeota*; (iv) Mx825 (Raskin *et al.*, 1994), specific for *Methanosaeta* spp. and (v) Sarci551 (Sorensen *et al.*, 1997), specific for *Methanosarcina* spp. were used in this study. Separate samples of the probes were fluorescently labelled at the 5′ end with Cy3 and Cy5. Hybridisations were carried out according to the method of Manz *et al.* (1992). Confocal laser scanning microscopy was employed for the examination of hybridised sections.

Radiotracer incubations with biofilm granules

Samples of S1 were used to investigate the pattern of substrate uptake in sludge samples by use of the radioactive tracer technique described by Andreasen and Nielsen (1997). Aliquots of 50 μl of granules (2.8 mg suspended solids (SS)) were added to 1.5 ml microcentrifuge tubes for radiotracer incubations. Sterilised biomass samples were used as negative controls. A panel of four different organic substrates were tested: [1(2)-^{14}C]acetic acid, sodium salt with a specific radioactivity of 59 mCi/mmol; D-[6-^{3}H]glucose with a specific activity of 33 Ci/mmol; L-[4,5-^{3}H]lysine monohydrochloride with a specific activity of 91 Ci/mmol and [D-glucose-1-^{14}C]lactose with a specific radioactivity of 56 mCi/mmol. Unlabelled substrates were pure grade chemicals from Sigma. The concentration of organic substrate in each tube was 20 mM to 100 mM, corresponding to a chemical oxygen demand of 5 g l^{-1} and a radioactivity of 3 to 10 μCi/mg of SS. Incubations were carried out at 15 °C and 37 °C for 8 h; a time-series experiment was achieved by stopping some incubations after 2 h and 4 h.

Preparation of biofilm samples for beta-imaging and FISH

After incubation, the organic substrate in the microcentrifuge tubes was immediately replaced with aliquots of a chilled 4% solution of paraformaldehyde in 1X PBS and incubated at 4 °C for at least 6 h. Samples were washed three times in PBS and stored in PBS:ethanol (1:1) at −20 °C, before preparation for sectioning as before. Sectioned samples were scanned for radioactivity using a micro-beta-imager. *In situ* hybridisations were carried out as before.

Results and discussion

Archaeal community structure of sludge samples

Clone library analysis of all six sludges investigated suggested a diverse range of archaeal communities among different sludge samples (Figure 1). No *Crenarchaeota*-like clones were detected in S2, S3 or S4, while high levels of these organisms were found in S1, S5 and S6 (70%, 56% and 59% of all archaeal clones, respectively).

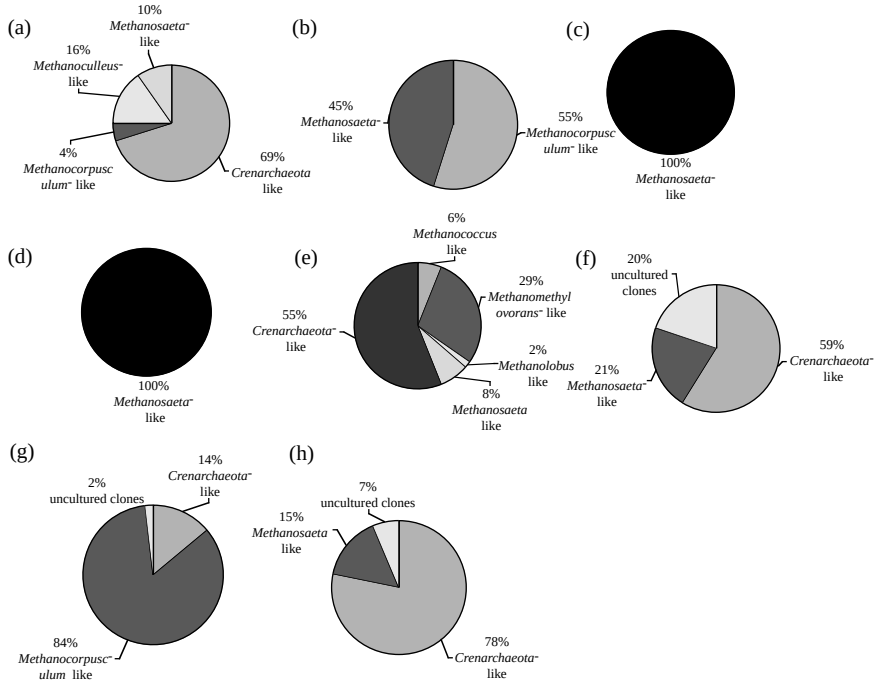

Figure 1 Composition of 16S rRNA gene clone libraries generated from (a) S1, (b) S2, (c) S3, (d) S4, (e), S5, (f) S6. The archaeal composition of R1 and R2 biomass at the conclusion of the trial is illustrated at (g) and (h)

Crenarchaeotal dynamics within bioreactor sludge as determined by TRFLP

Process-related population dynamics were evident, whereby temporal changes in the relative abundance of specific terminal restriction fragments (TRFs) were recorded (Figure 2). For example, a gradual decline in the relative abundance of *Methanosaeta*-like organisms (582 bp) was observed in parallel with an increased abundance of *Methanocorpusculum*-like peaks in R1 biomass (122 bp). An increased hydrogen-utilising (*Methanocorpusculum*) population, coupled with a reduced acetate-degrading (*Methanosaeta*-like), may reflect greater dependence on hydrogen-mediated methanogenesis, due to high organic loading under low-temperature conditions. Similar phenomena have been observed in previous low-temperature wastewater treatment trials (Collins *et al.*, 2005). Interestingly, a re-emergence of *Methanosaeta*-like TRFs was apparent by the conclusion of the trial, coinciding with a reduced organic loading.

With respect to the *Crenarchaeota*, *Thermophilus pendens* and *Sulfolobus*-like TRFs (235 bp) were detected throughout the reactor trial period (Figure 2). However, these organisms were more abundant in R2 at the conclusion of the experiment. Furthermore, the dynamics of the *Crenarchaeota*-like TRF peaks were directly related to operational perturbations and environmental changes in the R1 bioreactor. This observation suggests an active role for these members in the nutrient recycling processes of these biofilms.

FISH and micro-beta-imaging

FISH experiments illustrated a layered architectural topography for all granular biofilms investigated (Figure 3a). A layered structure has been reported by other authors (Sekiguchi *et al.*, 1999; Imachi *et al.*, 2000) and has been attributed to the concentric growth pattern of the anaerobic granules. There was generally good agreement between clone libraries and FISH analysis using the specific (*Methanosaeta*- and *Methanosarcina*-specific)

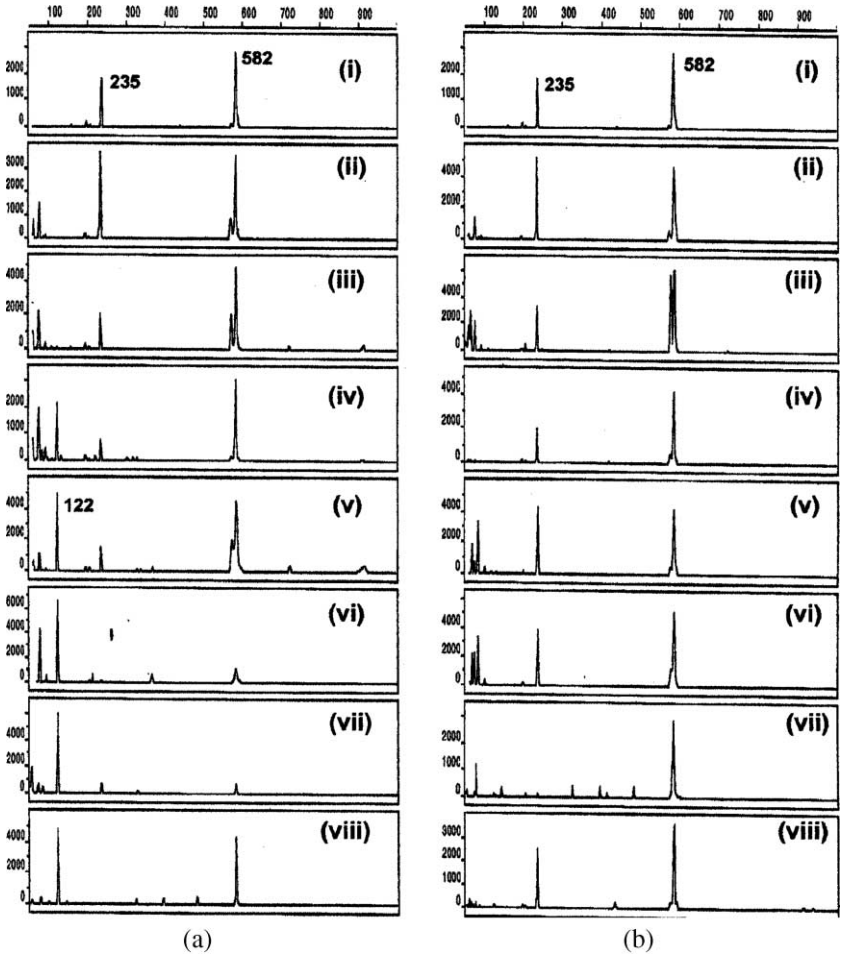

Figure 2 TRFLP profile illustrating crenarchaeotal dynamics (235 bp) of R1 (a) and R2 (b) on the following days: (i) 0, (ii) 21, (iii) 71, (iv) 218, (v) 309, (vi) 354, (vii) 409 and (viii) day 500

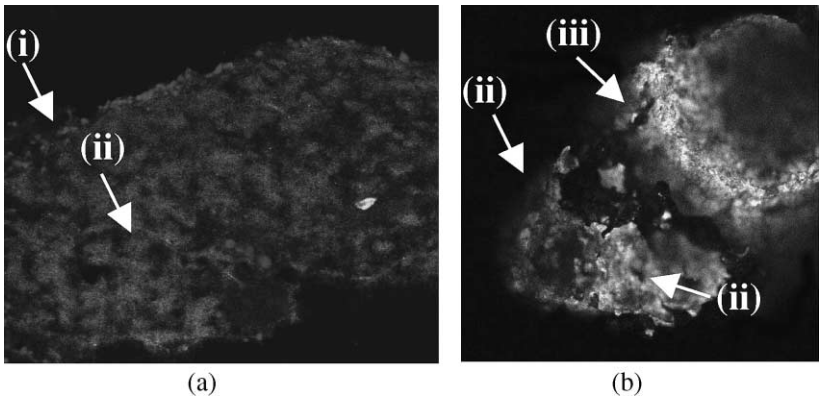

Figure 3 FISH illustrating: (a) the layered structure of (i) *Bacteria*, and (ii) *Archaea* in S4 granules; and (b) (i) *Bacteria*, (ii) *Archaea*, and (iii) a structured crenarchaeotal architecture in S5 biomass

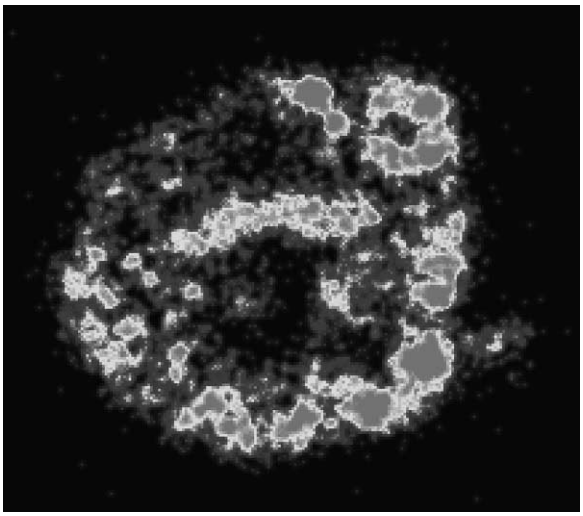

Figure 4 Beta-image generated from a cross-section of an acetate-fed S1 sludge granule

probes (data not shown). Furthermore, similar archaeal and crenarchaeotal dynamics were detected by FISH as by TRFLP for the samples obtained from R1 and R2. However, FISH experiments revealed that 40% of *Archaea* in S1 biomass were Cren 499-positive, thereby reflecting the overestimation of *Crenarchaeota*-like species by clone library analysis of S1. Thus, the value of this polyphasic approach (clone libraries, TRFLP and FISH) to ecological inventories is highlighted.

Beta-imaging suggested the localised accumulation of radioactivity within granules. For example, radioactivity was detected in the outer layers of biofilm sections from acetate incubations (Figure 4). This coincided with an abundance of filamentous *Methanosaeta* cells in juxtaposition with Cren499-positive cells around the outer layer of these granules. In addition, microcolonies of Cren499-positive cells, which coincided with radioactive accumulation, were observed throughout the topography of the biofilm sections (data not shown). While beta-imaging allowed determination of the spatial arrangement of active zones within anaerobic granules, the positive implication of groups, such as the *Crenarchaeota*, in specific substrate uptake may be better elucidated using microautoradiography and FISH (MAR-FISH).

Conclusions

The following conclusions may now be drawn: (i) *Crenarchaeota* are widespread and abundant in anaerobic wastewater treatment biofilms; (ii) the population dynamics of *Crenarchaeota*-like organisms may be linked to external stimuli and perturbations, thereby further indicating that these organisms have an important function; (iii) FISH and confocal microscopy illustrated a definite crenarchaeotal structure within granular biofilm architecture; (iv) this is preliminary data from ongoing beta-imaging work to elucidate the full *in situ* functionalism and ecophysiology of *Crenarchaeota* and anaerobic granular biofilm in general, and further experiments will achieve comprehensive data regarding uptake rates with a broader range of substrates. In summary, this is an important first step to understand the role of previously mysterious *Crenarchaeota* in biotechnologically important systems.

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